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Cross interference with TNF-α-induced TAK1 activation via EGFR-mediated p38 phosphorylation of TAK1-binding protein 1

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ABSTRACT

Transforming growth factor-α-activated kinase 1 (TAK1) has been widely recognized as a kinase that regulates multiple intracellular signaling pathways evoked by cytokines and immune receptor activation. We have recently reported that tumor necrosis factor-α (TNF-α) triggers internalization of epidermal growth factor receptor (EGFR) through a TAK1-p38α signaling pathway, which results in a transient suppression of the EGFR. In the present study, we investigated the pathway of intracellular signaling in the opposite direction. Ligand-induced activation of EGFR caused phosphorylation of the TAK1-binding proteins TAB1 and TAB2 in a TAK1-independent manner. EGFR-mediated phosphorylation of TAB1 was completely inhibited by a chemical inhibitor and siRNA of p38α. The phosphorylation of TAB1 was occurred at Ser-423 and Thr-431, the residues underlying the p38-mediated feedback inhibition of TAK1. In contrast, phosphorylation of TAB2 was sustained, and largely resistant to p38 inhibition. The inducible phosphorylation of TAB1 interfered with a response of EGF-treated cells to TNF-α-induced TAK1 activation, which led to the reduction of NF-κB activation. Collectively, these results demonstrated that EGFR activation interfered with TNF-α-induced TAK1 activation via p38-mediated phosphorylation of TAB1.

1. Introduction

Transforming growth factor-β-activated kinase 1 (TAK1) is one of the most characterized mitogen-activated protein kinase kinase kinase family (MAP3K) members and is activated by cellular stress, including tumor necrosis factor-α (TNF-α) and interleukin-1 [1–11]. TAK1 functions as an upstream stimulatory molecule of the c-Jun N-terminal kinase (JNK), p38, and nuclear factor-κB (NF-κB) signaling pathways. Gene targeting has clearly demonstrated that TAK1 participates in diverse cellular functions, including the activation and differentiation of T and B lymphocytes, and epidermal homeostasis involving the apoptosis of keratinocytes [12–17]. In addition, TAK1 has been shown to be involved in several pathogenic conditions, including inflammation, functional interaction with viral oncogene products, cancer metastasis, and myocardial hypertrophy [18–22].

The protein kinase activity of TAK1 is strictly controlled by TAK1-binding proteins (TAB) [6,11,23]. TAB1 binds to the N-terminal kinase domain of TAK1. TAB2 and the structurally similar TAB3 function as adaptor proteins to recruit TAK1 to TRAF2 (TNF-α receptor-associated factor) and TRAF6 in the TNF-α and interleukin-1 signaling pathways, respectively. We have reported that phosphorylation at Thr-187 is essential for TAK1 activation [24,25]. It has recently been shown that protein phosphatase 2A (PP2A) and PP6 are involved in the dephosphorylation of Thr-187 [26]. In addition, TAB1 participates in p38-mediated negative feedback regulation of the cytokine-induced TAK1 kinase activity, in which p38 directly phosphorylates TAB1 at Ser-423 and Thr-431 [10].

Epidermal growth factor receptor (EGFR) is a member of the ErbB receptor tyrosine kinase family and plays a critical role in a wide variety of cellular functions, including proliferation, differentiation, and apoptosis [27–30]. In addition to the true ligands, EGFR is transactivated by other extracellular stimuli, including agonists for G protein-coupled receptors, ion channels, integrins, and cellular stresses such as TNF-α, UV light and high osmolarity [31–37]. Recently obtained evidence has demonstrated that membrane-bound ligands of EGFR, such as TGF-α and heparin-binding EGF, are released and bind to the receptor in an extracellular fashion [38]. Moreover, evidence has been accumulating that TNF-α induces the rapid and transient internalization of EGFR with no obvious activation of its intracellular tyrosine kinase through the TAK1-p38 signaling pathway [39,40]. In fact, TNF-α actually suppresses the extracellular ligand-mediated activation of EGFR.

In tumor microenvironments and inflamed tissues, multiple cytokines and growth factors are expressed and coordinately regulate the pathogenic alterations. TNF-α and EGF are typical of these secreted ligands and their pathological functions have been extensively studied [41,42]. TNF-α plays a central role in inflammatory

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diseases, including rheumatoid arthritis and inflammatory bowel diseases [43]. In addition, attention has recently been focused on the role of TNF-α in tumor angiogenesis and metastasis [42]. On the other hand, overexpression, amplification and mutations of EGFR are involved in carcinogenesis and the progression of several types of cancer [27–30,41]. Anti-TNF-α and anti-EGFR agents have already been developed, and are clinically effective against these diseases, supporting the experimental analyses of these ligands and their receptors [43,44]. Cross talk between different signaling pathways is likely to be important for diverse cellular functions. Therefore, we have investigated the functional interactions of the TNF-α signal and EGFR signal to elucidate new biological processes.

MEKK1 and Raf-1, but not TAK1, are characterized as MAP3Ks underlying the EGFR-mediated activation of MAPKs including p38 [45,46]. As described above, p38 plays a key role in the feedback inhibition of TAK1 through phosphorylation of TAB1 [10]. In the present study, we have therefore investigated whether EGFR-induced, TAK1-independent activation of p38 triggers phosphorylation of TAB proteins and influences the TNF-α-induced activation of TAK1.

2. Materials and methods

2.1. Antibodies and reagents

An anti-phospho-TAK1 (Thr-187) antibody was described previously [25]. Other phospho-specific antibodies against p38 (Thr-180/Tyr-182), JNK (Thr-183/Tyr-185), ERK (Thr-202/Tyr-204), p65 (Ser-536), and EGFR (Tyr-845, Tyr-974 and Tyr-1068) and phospho-IκBα (Ser32/36) were purchased from Cell Signaling Technology. Anti-phospho-TAB1 (Thr-431, Ser-423, and Ser-438) antibodies were kindly provided by Dr. Phillip Cohen (The University Dundee). Antibodies against TAK1 (M-579), TAB1 (C-20), TAB2 (K-20), p38 (C-20-G), p65 (C-20-G), IκBα (C-21), EGFR (1005), PCNA (PC-10), Lamin B (C-20) and α-Tubulin (B-7) were purchased from Santa Cruz Biotechnologies. Recombinant human TNF-α and EGF were obtained from R&D System, and SB203580, SP600125, U0126 and AG825 from Merk Biosciences. Anisomycin was obtained from WAKO Pure Chemical Co. Ltd. 5Z-7-oxozeaenol, a selective TAK1 inhibitor, was a gift from Chugai Pharmaceutical Co. Ltd [47]. All the chemical inhibitors were dissolved in DMSO and the final concentration of DMSO was less than 0.1 %.

2.2. Expression vectors

Expression vectors for TAK1, TAB1 and its substitution mutant were reported previously [5,24,25]. The expression vector for TAB2 was kindly provided by Dr. Kunihiro Matsumoto (Nagoya University, Japan). EGFR cDNA was amplified by RT-PCR and inserted into pcDNA3.1 vector.

2.3. Cell culture and transfection

HeLa and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO2. HeLa and HEK293 cells were transfected with expression plasmids using LipofectAMINE and LipofectAMINE2000 reagents (Invitrogen), respectively.

2.4. Preparation of cell extracts

Whole cell lysates were prepared as described previously [4]. Cytoplasmic and nuclear extracts were prepared as described previously with some modifications [45]. In brief, cells were suspended in 400 μl of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and chilled on ice for 15 min. Next, 25 μl of 10% Nonidet P-40 was added and the suspension was vigorously vortexed for 10 s and kept on ice for 5 min. Cytoplasmic extracts were collected from the supernatants of centrifugation at 15,000 rpm for 5 min. The nuclear pellets were washed with 100 μl of buffer A and suspended in 50 μl of buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The mixture was kept on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at 15,000 rpm for 5 min.

2.5. Immunoblotting

Cell lysates or extracts were resolved by SDS-PAGE and transferred to an Immobilon-P nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) overnight at 4 °C and probed with primary antibodies as described above. Antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, anti-goat and anti-sheep IgG (DAKO), and visualized with the ECL system (Amersham Biosciences). Antibody reactions were carried out in the Can Get Signal solution (TOYOBO). The specificity of antibody was confirmed by the molecular weight of the band detected using BenchMark protein ladder (Invitrogen).

2.6. Immunoprecipitation

Cell lysates were diluted with an equal volume of dilution buffer (20 mM HEPES, pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After centrifugation, lysates were immunoprecipitated with anti-TAB2 antibody on ice for 1.5 h and then rotated with protein G-Sepharose (Amersham Bioscience) at 4 °C for 1.5 h. The Sepharose beads were washed three times with wash buffer (1:1 mixture of whole cell lysate buffer and dilution buffer).

2.7. RNA interference

Duplex siRNAs with two nucleotides overhanging at the 3′ end of the sequence were designed at iGENE Therapeutics and synthesized at Hokkaido System Science Co, Ltd. The target sequences were as follows; TAK1, UGGCUUAUCUUACACUGGA; EGFR, UGCUGGGUGCGGAAGUGAAAGAAUA; ErbB2, UGUCAGUAUCCAGGCUUUGUA; p38α, GCAUUACAACCAGACAGUUGAUAUU; p38α-m, CAUUACAACCCGCCAGUUGAUAUU; firefly luciferase (GL2), CGUACGCGGAAUACUUCGA. HeLa cells were transfected with siRNAs in a final concentration of 30–50 nM using Lipofectamine reagents. At 72 h post-transfection, cells were stimulated.

2.8. λ-Phosphatase reaction

TAK1 complex was immunoprecipitated with anti-TAB2 antibody from untreated or EGF-stimulated HeLa cells, and then incubated with λ-phosphatase (Upstate) at 37 °C for 30 min. Phosphatase activity was analyzed as mobility shift on SDS-PAGE.

2.9. Electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared from HeLa cells and probed with a ^{32}P-labeled oligonucleotide probe containing the consensus DNA-binding sequence of NF-κB [5]. The protein–DNA complexes were separated on 4% PAGE as described previously [5].

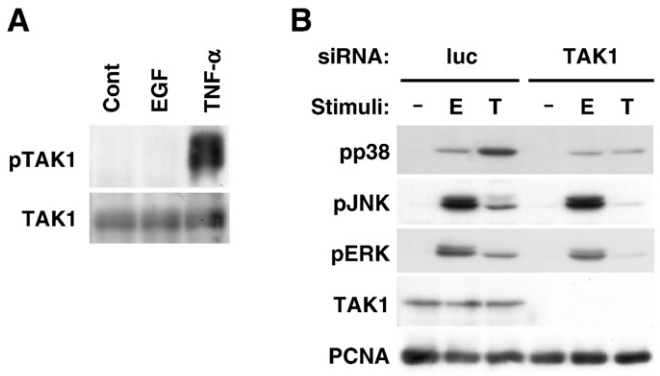

Fig. 1. TAK1 is dispensable in EGF signaling pathways. (A) HeLa cells were stimulated with 10 ng/ml EGF and 20 ng/ml TNF-α for 5 min. Whole cell lysates were immunoblotted with phospho-TAK1 and TAK1 antibodies. (B) Cells (2×10^5 cells/6-cm dish) were transfected with siRNA (40 nM) against TAK1 and luciferase (luc). At 72 h post-transfection, the cells were stimulated with or without EGF (E) or TNF-α (T) for 5 min. Cell lysates were immunoblotted with specific antibodies.

2.10. Luciferase assay

HEK293 cells were co-transfected with expression vectors for TAK1, TAB1, TAB2, EGFR and a luciferase reporter plasmid under the control of 4xκB site (Stratagene). pRL-EF1αp (kindly provided by Dr. Tsuda) was also transfected. The luciferase activity was measured by using the Dual-Luciferase reporter assay system (Promega).

3. Results

3.1. EGF-induced MAPK activation is independent on TAK1

TAK1 has been shown to regulate MAPK pathways from cytokine receptors. However, the role in growth factor signaling pathways remains to be investigated. As reported previously, treatment of HeLa cells with TNF-α induced rapid phosphorylation of TAK1 at Thr-187 (Fig. 1A). In contrast, no TAK1 phosphorylation was detected in response to EGF (Fig. 1A). In addition, although knockdown of TAK1 expression by siRNA inhibited TNF-α-induced MAPK activation, TAK1 was dispensable in the EGF-induced signaling pathways (Fig. 1B).

3.2. EGF-induced modification of TAB1 and TAB2

Treatment of HeLa cells with EGF induced receptor tyrosine activity of EGFR and activation of its downstream signaling pathways leading to MAPKs (Fig. 2A). Here, we newly identified modifications of TAB1 and TAB2 as shifts in mobility on SDS-PAGE upon stimulation with EGF (Fig. 2A). The mobility shift of TAB1 was rapidly induced within 5 min and then returned to the control level at 30 min. On the other hand, the mobility shift of TAB2 sustained for at least 30 min (Fig. 2A). To investigate the pathway leading to TAB1 and TAB2 upon EGF stimulation, we examined the effect of PD153035 and AG825, potent and selective tyrosine kinase inhibitors for EGFR and ErbB2, respectively. Fig. 2B shows that PD153035 inhibited the EGF-induced mobility shift of TAB1 and TAB2 as well as phosphorylation of EGFR and p38 (Fig. 2B). In contrast, AG825 had no inhibitory effects on these cellular responses (Fig. 2B). Similarly, an RNAi experiment demonstrated that EGFR, but not ErbB2, is essential for the EGF-induced cellular responses (Fig. 2C). These results indicate that the modification of TAB1 and TAB2 was mediated by tyrosine kinase activity of EGFR homodimer.

3.3. p38α is involved in phosphorylation of TAB1 and TAB2

To identify the kind of modifications that occurred on TAB1 and TAB2, we first investigated the possibility of phosphorylation. The TAK1 complex was immunoprecipitated with anti-TAB2 antibody from cells untreated or treated with EGF for 10 min and immunoprecipitates were incubated with λ-phosphatase *in vitro*. Fig. 3A shows that the reduced mobility was completely restored by λ-phosphatase, indicating that phosphorylation is involved, at least in part, in EGF-induced modification of TAB1 and TAB2.

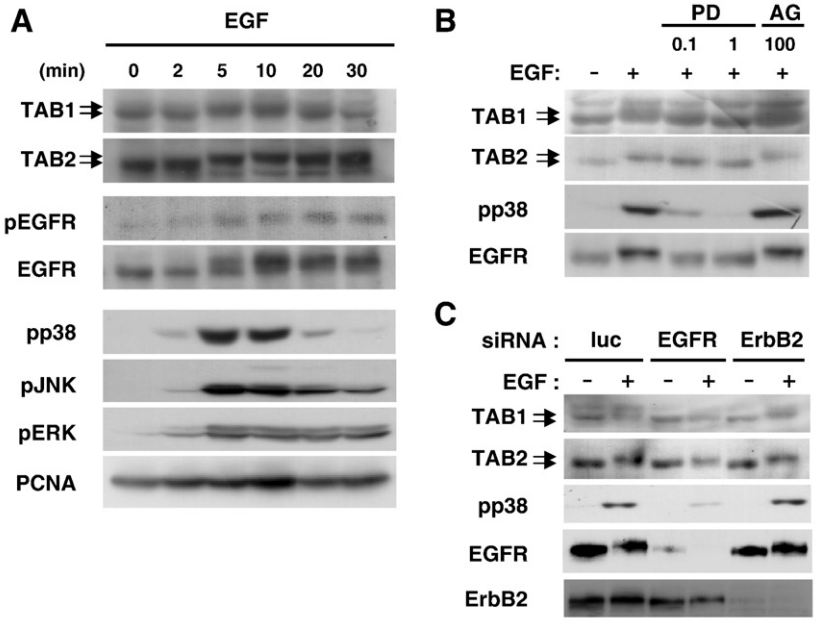

Fig. 2. EGF-induced modification of TAB1 and TAB2. (A) HeLa cells were stimulated with 10 ng/ml EGF for the indicated periods. Whole cell lysates were immunoblotted with specific antibodies indicated on the left side of each panel. pY means the tyrosine-phosphorylated form of EGFR. Phosphorylation of p38, JNK, and ERK was analyzed by their phospho-specific antibodies. (B) HeLa cells were pretreated with the indicated concentrations (μM) of PD153035 (PD), AG825 (AG) for 30 min and then stimulated with EGF for 10 min. Whole cell lysates were immunoblotted with specific antibodies. (C) Cells were transfected with siRNA (50 nM) against EGFR, ErbB2 and luciferase (luc). At 72 h post-transfection, the cells were stimulated with EGF for 10 min. Cell lysates were immunoblotted with specific antibodies.

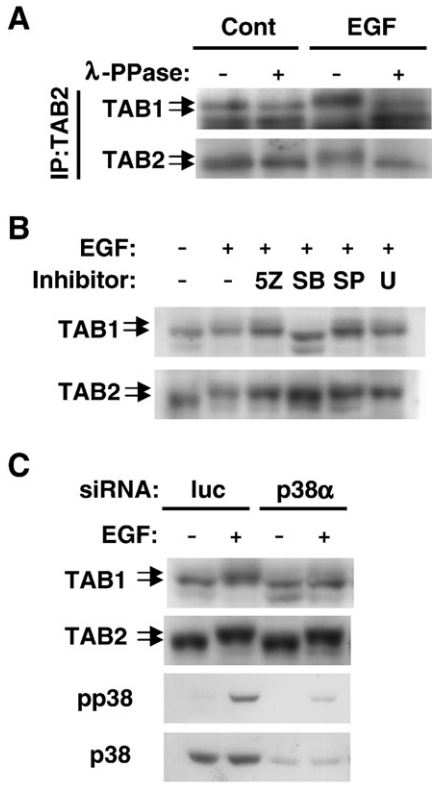

Fig. 3. Role of p38α in EGF-induced phosphorylation of TAB1 and TAB2. (A) HeLa cells were stimulated with EGF for 5 min. TAB2 was immunoprecipitated (IP) from untreated or EGF-stimulated cells and then incubated with λ-phosphatase (λ-PPase) *in vitro*. The shift in mobility was analyzed by immunoblotting with antibodies against TAB1 and TAB2. (B) HeLa cells were pretreated with 5Z-7-oxozeaenol (0.3 μM), SB203580 (20 μM), SP600125 (20 μM), or U0126 (10 μM) for 30 min and then stimulated with EGF for 10 min. Cell lysates were immunoblotted with antibodies against TAB1 and TAB2. (C) Cells were transfected with siRNA (50 nM) against p38α and luciferase (luc). At 72 h post-transfection, the cells were stimulated with EGF for 10 min. Cell lysates were immunoblotted with antibodies against TAB1, TAB2, phospho-p38 and p38.

We next examined the effects of chemical inhibitors for downstream kinases. Fig. 3B shows that phosphorylation of TAB1 was completely inhibited by pretreatment with SB203580, an inhibitor for p38α and p38β, while phosphorylation of TAB2 was only partially prevented by the inhibitor. Pretreatment with SP600125 and U0126, inhibitors for JNK and MEK1/2 (mitogen-activated protein kinase/extracellular signal regulated kinase kinase), respectively, did not block phosphorylation of TAB1 and TAB2 (Fig. 3B). In addition, phosphorylation of TAB1 and TAB2 was not affected by 5Z-7-oxozeaenol, indicating that the EGF-induced signal to TAB proteins was not dependent on the kinase activity of TAK1 (Fig. 3B). Similar results were obtained in experiments using siRNA against p38α. Fig. 3C shows that knockdown of p38α decreased phosphorylation of TAB1, but only slightly inhibited phosphorylation of TAB2. These results demonstrated that p38α is a critical mediator in EGF-induced TAB1 phosphorylation; however, its contribution to TAB2 phosphorylation is limited.

3.4. Identification of phosphorylation sites on TAB1

To identify the phosphorylation sites of TAB1, we performed immunoblotting with three phospho-specific anti-TAB1 antibodies against Thr-431, Ser-423 and Ser-438, which are the sites underlying p38-mediated feedback phosphorylation of TAB1 [10]. Unfortunately, phosphorylation of TAB1 in cell lysates could not be detected. In addition, anti-TAB1 antibody was unavailable for immunoprecipitation (data not shown). In contrast, TAB1 was co-immunoprecipitated with anti-TAB2 antibody (Fig. 4). Fig. 4A shows that TAB1 was strongly phosphorylated at Ser-423 and Thr-431 from 5 min to 20 min after stimulation with EGF. This was similar to the time course of the mobility shift on SDS-PAGE (Fig. 2A), suggesting that the EGF-induced mobility shift of TAB1 was mainly dependent on phosphorylation at Thr-431 and Ser-423. The phosphorylation was completely inhibited by SB203580, indicating a role for p38 (Fig. 4B). In contrast, phosphorylation at Ser-438 was stably detected under the basal conditions and was not induced by EGF. In addition, the basal phosphorylation was resistant to treatment with SB203580 (Fig. 4B). This is consistent with the observations that Ser-438 phosphorylation is not suppressed by SB203580 or in p38α MAPK$^{-/-}$ MEFs [10], and that JNK and ERK are possible kinases catalyzing Ser-438 phosphorylation [48]. Collectively, these results demonstrated that EGF induces phosphorylation of TAB1 at Ser-423 and Thr-431 in a p38-dependent manner.

3.5. Transient suppression of TAK1 in EGF-treated cells

The inducible phosphorylation of TAB1 at the sites for feedback inhibition raises the possibility that pretreatment with EGF inhibits activation of TAK1 by TNF-α. To test this hypothesis, HeLa cells were pretreated with EGF for the indicated periods (0–30 min), and then stimulated with TNF-α for another 5 min in the presence of EGF. Without the pretreatment, the 5 min TNF-α stimulation induced phosphorylation of TAK1 at Thr-187, an indicator for the activation status of TAK1 (Fig. 5A). Interestingly, the TNF-α-induced phosphorylation of TAK1 was impaired by the pretreatment with EGF (Fig. 5A). Notably, a significant reduction in the activation of TAK1 was observed in cells pretreated with EGF for 5–20 min, which corresponded to the timing of the phosphorylation of TAB1. We next investigated the kinetics of TNF-α-induced TAK1 phosphorylation after the 10 min EGF

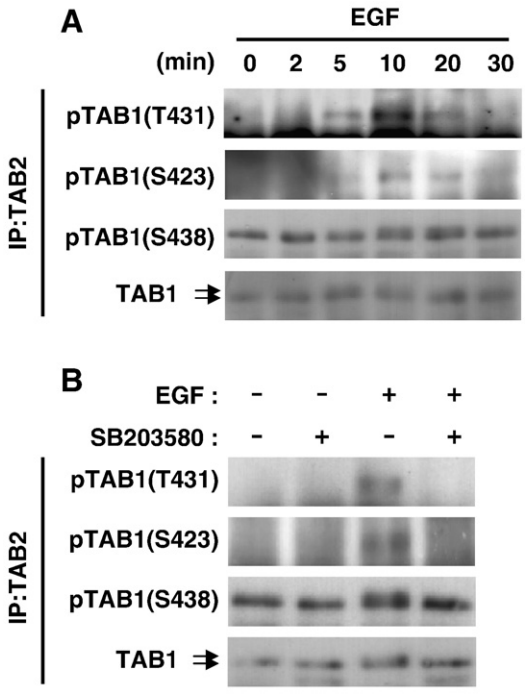

Fig. 4. EGF-induced phosphorylation of TAB1 at Ser-423 and Thr-431. (A) HeLa cells were stimulated with 10 ng/ml EGF for the indicated periods. The TAK1 complex was immunoprecipitated (IP) with anti-TAB2 antibody and then immunoblotted with antibodies against total TAB1 and phospho-specific TAB1 antibody corresponding to Thr-431, Ser-423 and Ser-438. (B) HeLa cells were pretreated with or without SB203580 (20 μM) for 30 min and then stimulated with EGF for 10 min. Cell lysates were immunoprecipitated (IP) as described in A.

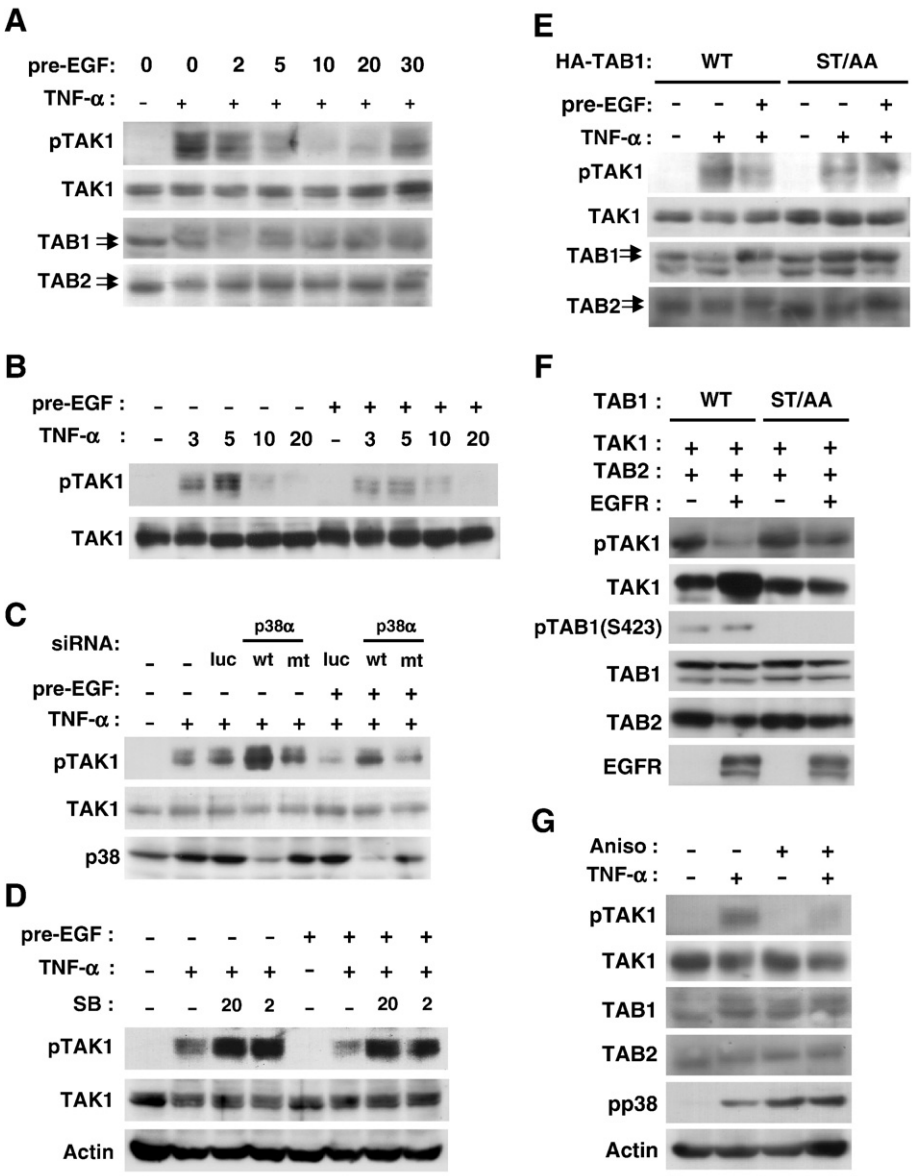

Fig. 5. Transient suppression of TAK1 activation by EGF. (A) HeLa cells were pretreated with EGF for the indicated period and then stimulated with TNF-α for another 5 min. Whole cell lysates were immunoblotted with anti-phospho-TAK1, anti-TAK1, anti-TAB1 and anti-TAB2 antibodies. (B) HeLa cells were treated with or without EGF for 10 min and post treated with TNF-α for the indicated time. Whole cell lysates were immunoblotted with anti-phospho-TAK1, anti-TAK1 antibodies. (C) HeLa cells were transfected with or without siRNA (30 nM) against wild type (wt) or mutant (mt) p38α, and luciferase (si-luc). At 72 h post-transfection, the cells were stimulated with or without EGF (for 10 min), and then stimulated with TNF-α in the presence of EGF for another 5 min. Cell lysates were immunoblotted with antibodies against phospho-TAK1, TAK1 and p38. (D) HeLa cells were pretreated with 20 μM and 2 μM of SB203580 for 30 min. Cells were stimulated with or without EGF for 10 min and then stimulated with TNF-α for another 5 min. Whole cell lysates were immunoblotted with anti-phospho-TAK1, anti-TAK1 and anti-actin antibodies. (E) HeLa cells were transfected with expression vectors for wild type HA-TAB1 or its substitution mutant (Ser-423-Ala and Thr-431-Ala, AA). At 24 h post-transfection, cells were stimulated with or without EGF for 10 min, and then stimulated with TNF-α in the presence of EGF for another 2.5 min. Cell lysates were immunoblotted with antibodies against phospho-TAK1, TAK1, TAB1 and TAB2. (F) HEK293 cells (1.5×10^6/6 cm dish) were transfected with expression vectors for Flag-TAK1 (0.5 μg), HA-TAB1 (0.5 μg) or its substitution mutant (Ser-423-Ala and Thr-431-Ala, AA) (0.5 μg), TAB2 (0.5 μg) and/or EGFR (0.5 μg). At 24 h post-transfection, cell lysates were immunoblotted with antibodies against phospho-TAK1, TAK1, TAB1, phospho-TAB1 (Ser-423), TAB2 and EGFR. (G) HeLa cells were pretreated with 60 μM of anisomycin for 15 min and then stimulated with TNF-α for another 5 min. Whole cell lysates were immunoblotted with anti-phospho-TAK1, anti-TAK1, anti-TAB1, anti-TAB2, anti-phospho-p38 and anti-actin antibodies.

pretreatment (Fig. 5B). Without the pretreatment, rapid and transient TAK1 phosphorylation was detected within 10 min after TNF-α exposure. The kinetics of TAK1 phosphorylation was not affected by the EGF pretreatment, although the intensity was significantly decreased (Fig. 5B).

3.6. TAK1 is negatively regulated by p38-mediated TAB1 phosphorylation

To elucidate the role of p38 in this suppression, we examined the effects of RNA interference (RNAi). HeLa cells were transfected with wild type p38α siRNA, its mutant (p38-mt) and control luciferase (luc) siRNA. At 72-h post-transfection, the specific knockdown of p38 expression was confirmed by immunoblotting. We tried to examine the role of p38 in the EGF-induced suppression of TAK1 in these transfected cells. As reported previously, inactivation of p38 canceled the feedback inhibition of TAK1, which resulted in enhanced TAK1 phosphorylation upon TNF-α stimulation (Fig. 5C, lane 4). EGF-induced suppression of TAK1 was reproducibly detected in cells transfected with control siRNA (Fig. 5C, lane 6). Moreover, the TNF-α-induced TAK1 activation was clearly improved by p38α RNAi (Fig.

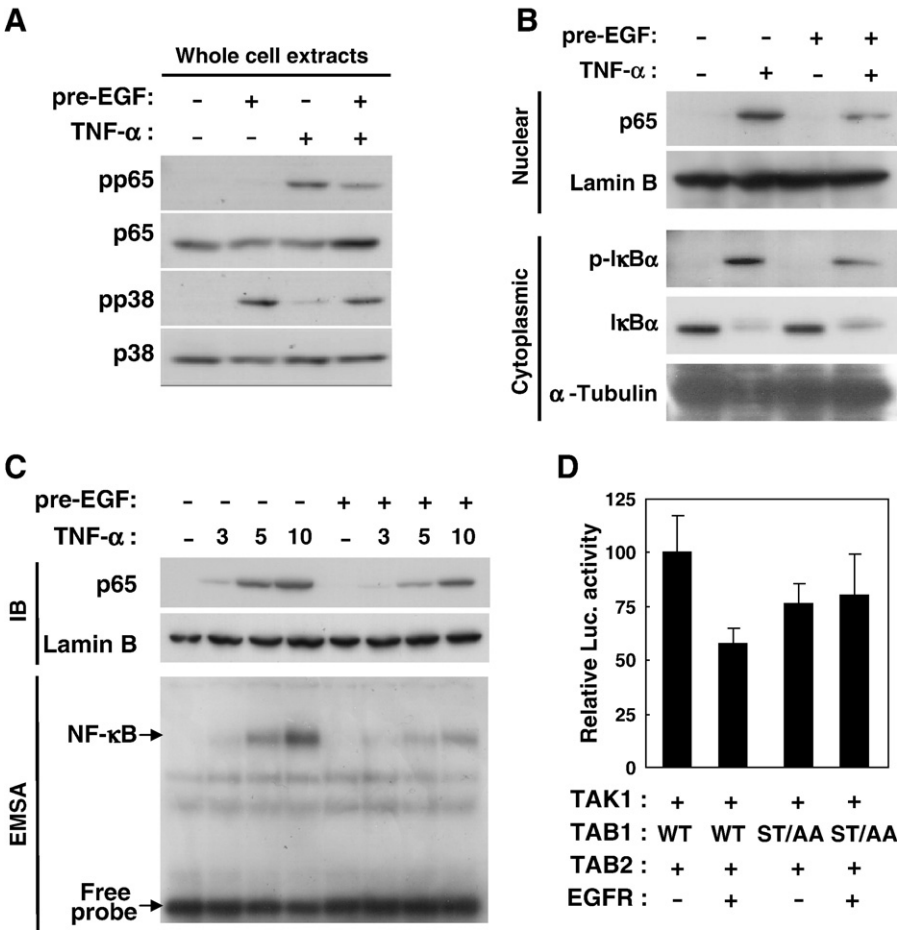

Fig. 6. EGF-induced suppression of TAK1 activity elicits reduced NF-κB activation. (A) HeLa cells were pretreated with EGF for 10 min and then stimulated with TNF-α for another 2 min. Whole cell lysates were immunoblotted with antibodies against phospho-p65, p65, phospho-p38 and p38. (B) HeLa cells were pretreated with EGF for 10 min and then stimulated with TNF-α for another 5 min. Nuclear extracts were immunoblotted with antibodies against p65 and Lamin B. Cytoplasmic extracts were immunoblotted with antibodies against phospho-IκBα, IκBα and α-Tubulin. (C) HeLa cells were treated with or without EGF for 10 min and post treated with TNF-α for the indicated time. Nuclear extracts were examined by immunoblotting (IB) and gel mobility shift assay (EMSA). (D) HEK293 cells (2.0×10^4/12-well plate) were transfected with expression vectors for Flag-TAK1 (0.5 μg), HA-TAB1 (0.5 μg) or its substitution mutant (ST/AA) (0.5 μg), TAB2 (0.5 μg) and EGFR (0.5 μg), together with luciferase reporter plasmids driven by NF-κB and EF1α promoter. Twenty-four hour after transfection, luciferase activities were determined. Data are the mean ± S.D. of triplicates from a representative experiment.

5C, lane 7). A similar result was obtained in an experiment using a p38 specific inhibitor SB203580. The inhibitor at 20 μM and 2 μM abrogated the EGF-induced suppression of TAK1 phosphorylation (Fig. 5D, lanes 7, 8). In addition, overexpression of TAB1-ST/AA, which has a substitution mutation in the p38-mediated phosphorylation sites (Ser-423 and Thr-431), rescued the EGF-induced suppression of TAK1 (Fig. 5E). Moreover, an overexpression experiment confirmed that phosphorylation of TAK1 by wild type TAB1 and TAB2 were significantly inhibited by co-expression of EGFR (Fig. 5F). In contrast, TAB1-ST/AA mutant-induced phosphorylation of TAK1 was resistant to the suppression by EGFR (Fig. 5F). These results demonstrated that EGF-induced down-regulation of TAK1 is mediated through p38-induced phosphorylation of TAB1 at Ser-423 and Thr-431. Furthermore, anisomycin induced both p38 activation and suppression of TNF-α-induced activation of TAK1 (Fig. 5G), suggesting that the suppression is a common mechanism for p38-activting agents.

3.7. EGF inhibits TNF-α-induced NF-κB activation

TNF-α and EGF have been shown to affect similar intracellular signaling pathways including MAPKs. However, the transcription factor NF-κB is specifically activated by TNF-α. We have reported that TNF-α-induced phosphorylation of NF-κB p65 at Ser-536 is mediated through the TAK1 complex [49,50]. Therefore, we investigated phosphorylation of p65 to evaluate the effect of the EGFReceived in revised forminduced suppression of TAK1 on its downstream signaling pathway. Cells were treated with EGF for 10 min and subsequently treated with TNF-α for another 2 min. TNF-α-induced phosphorylation of NF-κB was reduced by the EGF pretreatment (Fig. 6A). Similarly, the EGF pretreatment inhibited nuclear translocation of p65, phosphorylation and degradation of IκBα (Fig. 6B). We next investigated the kinetics of TNF-α-induced NF-κB activation after the 10 min EGF pretreatment. Western blot analysis and EMSA demonstrated that NF-κB activation in EGF-treated cells was reduced at all the time points tested (Fig. 6C). In addition, TAK1 complex (TAK1, TAB1 and TAB2)-induced NF-κB-dependent reporter gene expression was suppressed by EGFR in HEK293 (Fig. 6D). Similar to the effect on TAK1 (Fig. 5D), the substitution TAB1 mutant was resistant to the suppression by EGFR. These results indicate that the reduced activation of TAK1 was correlated with the selective suppression of TNF-α-induced activation of NF-κB.

4. Discussion

EGFR is activated by natural ligands as well as other extracellular stimuli. It has recently been demonstrated that EGFR plays a role in the

TNF-α-induced proliferation and motility of hepatocytes and mammary epithelial cells. Gefitinib, a selective EGFR tyrosine kinase inhibitor, blocks TNF-α-induced metastatic properties in mouse hepatocellular carcinoma cells [35–37]. Moreover, we have recently reported that TNF-α suppresses extracellular EGF responses through internalization of the EGFR [40], a process in which the TAK1-p38α signaling pathway regulates phosphorylation of EGFR at unidentified sites other than the known major intracellular tyrosine residues (Figs. 1 and 7). In the present study, we demonstrated the pathway of suppressive signaling in the opposite direction. EGFR-mediated activation of p38α induced phosphorylation of TAB1 at the feedback sites in a TAK1-independent manner, which resulted in an attenuation of TNF-α-induced TAK1 activation. Fig. 1B showed that TAK1 was dispensable in the EGF-induced p38 activation. Among the other MAP3Ks, MEKK1, 2, 3, and 4 have been shown to mediate MAPK activation by EGF [41,42]. Collectively, these results indicate that the TNF-α and EGFR signaling pathways interfere with each other, where p38α is a common mediator connecting both directions of suppressive signals.

TAK1 catalytic activity is controlled by the TAK1-binding proteins TAB1, TAB2 and TAB3. In an overexpression experiment, TAB1 was the most potent activating protein among TAK1-binding proteins. The role of TAB1 in TAK1 activation was confirmed in TAB1$^{-/-}$ mouse embryonic fibroblastic cells (MEFs), in which cytokine-induced phosphorylation of TAK1 at Thr-187 was reduced as compared with that observed in wild type MEFs [48,51]. In addition to this activating potential, it is interesting that TAB1 is also involved in the negative feedback regulation of TAK1 activity [10,48]. The cytokine-induced activation of p38 via TAK1 resulted in the inducible phosphorylation of TAB1 at Ser-423 and Thr-431. In the present study, we found that the EGFR-mediated signal mimics the p38α-TAB1 inhibitory pathway to prevent cytokine-induced TAK1 activation. However, a critical difference between the feedback inhibition and EGF-induced inhibition is whether the p38-TAB1-mediated inhibitory signal is dependent on TAK1 kinase activity or not. Therefore, this is the first report demonstrating a preventive mechanism for cytokine-induced TAK1 activation. It has recently been reported that other phosphorylation sites in the activation loop of TAK1 (Thr-178 and Thr184) play regulatory role in TAK1 activation [52]. Study focused on these sites will provide more information for the suppression and negative feedback inhibition of TAK1 by the p38-TAB1 pathway.

p38α MAPK is activated mainly by the upstream MAPK kinases (MAP2Ks), MKK3 and MKK6. As compared to the limited number of MAP2Ks, many MAP3Ks have been shown to relay p38 signaling in response to a wide variety of cellular stimuli. Although TAK1 plays a central role in signaling from pro-inflammatory cytokine receptors and immunoreceptors, it is unlikely to participate in a large number of p38-activating stimuli. Therefore, a preventive mechanism for TAK1 activation via p38-TAB1 may generally occur in response to cellular p38-activating stimuli including EGF. It is further interesting that TAB1 has been shown to directly activate p38 in a MKK- and TAK1-independent manner [53]. TAB1β, a splicing variant of TAB1 which lacks the TAK1-binding region but has the p38-binding region, has been shown to be involved in the invasiveness of breast cancer cells [54]. These observations suggest that p38-mediated phosphorylation of TAB1 affects TAK1-independent functions of TAB1. Therefore, the p38-TAB1 interaction may play a key role in TAK1-dependent and -independent cellular events.

TAB2 and TAB3 are structurally related and suggested to have a redundant function [6,11]. These proteins are also phosphorylated in response to TNF-α and IL-1. Mendoza et al. recently identified the cytokine-induced phosphorylation sites in TAB2 (Ser-372, Ser-524 and Ser-582) and TAB3 (Ser-60, Thr-404 and Ser-506) [48]. Ser-372 and Ser-524 in TAB2 structurally correspond to Thr-404 and Ser-506 in TAB3. However, it is quite interesting that although all the sites in TAB3 and Ser-582 in TAB2 are phosphorylated through p38α, the other sites in TAB2 are independent of p38α. This is correlated with our finding that EGF-induced phosphorylation of TAB2 was largely resistant to inactivation of p38α. In spite of the identification of phosphorylation sites, the functional importance of TAB2 and TAB3 in the feedback TAK1 inhibition is

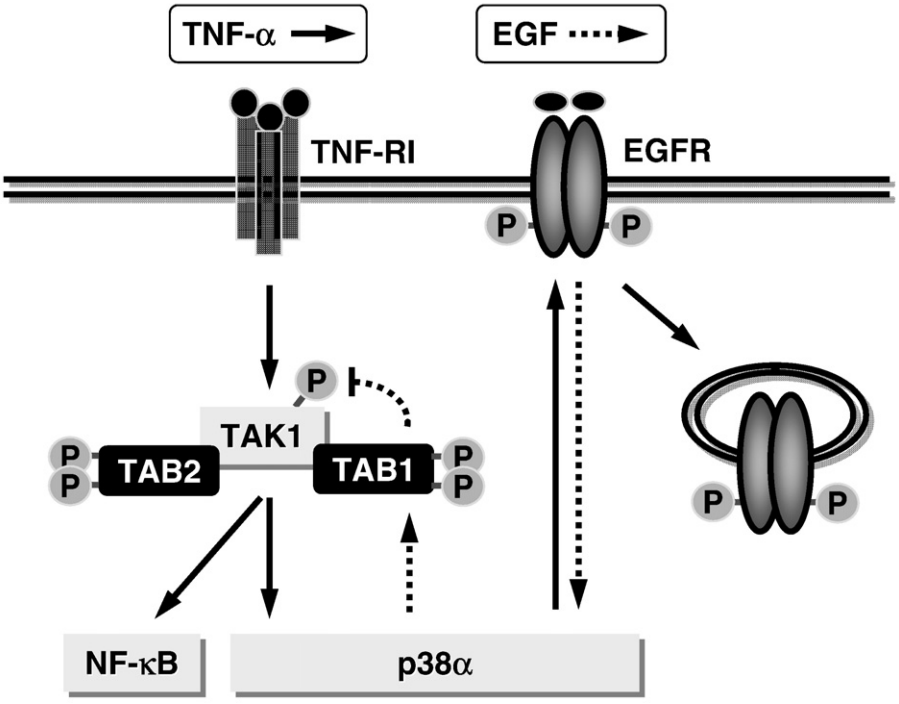

Fig. 7. A schematic diagram for cross interference between TNF-α and EGF signaling pathways. The TNF-α-induced signaling pathway from TNF receptor type I (TNF-RI) to EGFR is shown with a solid arrow. EGFR is phosphorylated at unidentified residues through p38α, which leads to endocytosis. The EGF-induced signaling pathway from EGFR is shown with a dashed arrow. The EGFR-mediated signal prevents TNF-α-induced phosphorylation of TAK1 through p38α-mediated phosphorylation of TAB1.

still unknown. The kinetics of the cytokine-induced phosphorylation of TAB2 and TAB3 was similar to that of TAB1. This is the case in the EGF-induced phosphorylation of TAB2. Although we have not yet addressed the phosphorylation of TAB3 in EGF-treated cells, these results suggest a possible involvement of TAB2 and TAB3 in cytokine-induced negative feedback control and the EGF-induced prevention of TAK1 activation.

The physiological function of the cross interference between the TNF-α and EGF signaling pathways is not yet understood. It is important that these suppressive signals are transient. In the case of TNF-α-induced EGFR suppression, p38-mediated internalization is triggered within 10 min, and then EGFR is dephosphorylated to recycle back to the cell surface at 60 min. The recycled EGFR is able to respond again to extracellular ligands. In the present study, we showed that p38-TAB1-mediated suppression of TAK1 was detected mainly at 5–20 min after the EGF stimulation. These results raise the possibility that, in tissue microenvironments presenting multiple kinds of extracellular ligands, intracellular signals triggered by one receptor interfere with signals from another receptor to predominantly incorporate the earlier signals. In any case, more investigation is needed to understand the physiological and pathological significance of the transient cross interference of intracellular signals.

In summary, we have found a novel intracellular communication network between the TNF-α and EGF signaling pathways. These findings raise the possibility that cellular responses to more than one ligand differ in their order of stimulation. Therefore, systematic analyses of short-term lag stimulation will provide new insight into the biological responses of the cell.

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